PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

To:

see form PCT/ISA/220

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY
(PCT Rule 43*bis*.1)

Date of mailing *(day/month/year)* see form PCT/ISA/210 (second sheet)	
Applicant's or agent's file reference see form PCT/ISA/220	**FOR FURTHER ACTION** See paragraph 2 below
International application No. PCT/GB2004/005317	International filing date *(day/month/year)* 17.12.2004
Priority date *(day/month/year)* 19.12.2003	
International Patent Classification (IPC) or both national classification and IPC C07K14/80, C12N15/67, G01N33/50, C12N5/10	
Applicant AMERSHAM BIOSCIENCES UK LIMITED	

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☒ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. **FURTHER ACTION**

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA"). However, this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1*bis*(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of three months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

3. For further details, see notes to Form PCT/ISA/220.

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WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2004/005317

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   ☐ This opinion has been established on the basis of a translation from the original language into the following language , which is the language of a translation furnished for the purposes of international search (under Rules 12.3 and 23.1(b)).

2. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application and necessary to the claimed invention, this opinion has been established on the basis of:

   a. type of material:

   ☒ a sequence listing

   ☐ table(s) related to the sequence listing

   b. format of material:

   ☒ in written format

   ☒ in computer readable form

   c. time of filing/furnishing:

   ☒ contained in the international application as filed.

   ☒ filed together with the international application in computer readable form.

   ☐ furnished subsequently to this Authority for the purposes of search.

3. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

4. Additional comments:

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY

International application No.
PCT/GB2004/005317

Box No. II Priority

1. ☐ The following document has not been furnished:

   ☐ copy of the earlier application whose priority has been claimed (Rule 43*bis*.1 and 66.7(a)).

   ☐ translation of the earlier application whose priority has been claimed (Rule 43*bis*.1 and 66.7(b)).

   Consequently it has not been possible to consider the validity of the priority claim. This opinion has nevertheless been established on the assumption that the relevant date is the claimed priority date.

2. ☐ This opinion has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid (Rules 43*bis*.1 and 64.1). Thus for the purposes of this opinion, the international filing date indicated above is considered to be the relevant date.

3. ☒ It has not been possible to consider the validity of the priority claim because a copy of the priority document was not available to the ISA at the time that the search was conducted (Rule 17.1). This opinion has nevertheless been established on the assumption that the relevant date is the claimed priority date.

4. Additional observations, if necessary:

Box No. V Reasoned statement under Rule 43*bis*.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	1-47
	No:	Claims	none
Inventive step (IS)	Yes:	Claims	1-47
	No:	Claims	none
Industrial applicability (IA)	Yes:	Claims	1-47
	No:	Claims	none

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

1. Introduction

The present communication refers to the following documents (D) cited in the international search report.

D1: CHANDRA DHYAN ET AL: "Early mitochondrial activation and cytochrome c up-regulation during apoptosis." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 277, no. 52, 27 December 2002 (2002-12-27), pages 50842-50854, XP002320034 ISSN: 0021-9258

D2: YU TIANNING ET AL: "A mutational epitope for cytochrome c binding to the apoptosis protease activation factor-1" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 276, no. 16, 20 April 2001 (2001-04-20), pages 13034-13038, XP002320035 ISSN: 0021-9258

The application relates to a fusion protein comprising (i) a marker such as e.g. green fluorescent protein (GFP) and (ii) a modified cytochrome c protein, wherein said modified cytochrome c is still located in the mitochondria but has a "reduced ability to induce apoptosis in a living cell". Cells expressing said protein can be used to measure the degree of apoptosis by determining the amount of marked cytochrome released from the mitochondria into the cytoplasm. Advantageously, in a host cell which expresses said protein apoptosis is not initiated by the overexpression of the cytochrome c, i.e. the cells can be easily cultivated and form stable cell lines (see e.g. page 5, lines 16-27). The cytochrome c is preferably modified in that it binds Apaf-1 less efficiently than the wild type cytochrome c, wherein numerous positions and amino acid changes having said effect are known from the prior art and listed in the application.

According to D1 cells are transfected with a plasmid encoding a cytochrome c-GFP fusion protein in an ecdysone-inducible system. It is shown that the amount of cytochrome c translocated to the mitochondria is increased and it is accumulated in the mitochondria thereby resulting in the induction of apoptosis.

D2 discloses numerous mutations including residues 7, 25, 39, 62-65, and 72, which are

involved in the cytochrome c - Apaf-1 interaction, wherein mutations at position 72 eliminate the ability of cytochrome c to initiate apoptosis and the other mutations reduce said ability.

2. Novelty and inventive step

The subject-matter of all claims is novel and inventive.

The application relates to a fusion protein comprising (i) a marker such as e.g. green fluorescent protein (GFP) and (ii) a "modified" cytochrome c protein, wherein said "modified" cytochrome c is still located in the mitochondria but has a "reduced ability to induce apoptosis in a living cell". Cells expressing said protein can be used to measure the degree of apoptosis by determining the amount of marked cytochrome released from the mitochondria into the cytoplasm.

The prior art already discloses fusion proteins of cytochrome c and a marker such as GFP (see e.g. D1, page 50844, left-hand column, fourth paragraph). However, the cytochrome c is not "modified". If wild-type cytochrome c is overexpressed, apoptosis is initiated, i.e. transfected cells do not form stable cell lines (see e.g. D1). Said problem is overcome e.g. in D1 by using an ecdysone-inducible system, which allows to control cytochrome c expression.

Hence, the objective problem underlying the present application can be formulated as the provision of an alternative system for measuring the degree of apoptosis in a host cell.

The solution consisting in the provision of a fusion protein comprising a marker protein and a "modified" cytochrome c protein, wherein said "modified" cytochrome c is still located in the mitochondria but has a "reduced ability to induce apoptosis in a living cell", is not obvious in view of any of the cited documents alone or in combination. Although the structure and effect of both parts of the fusion protein were known, namely markers such as GFP on the one hand and mutated forms of cytochrome c which are not capable of initiating apoptosis on the other hand (see e.g. D2), the skilled practitioner had no reason to combine both documents.

The above-mentioned arguments apply not only for the fusion protein (claims 1-20), but also for the corresponding nucleic acid sequence(21-26), vectors (claims 27-30), host cells (claims 31-37), and methods for detecting apoptosis (claims 38-47)

Re Item VIII

The terms "cytochrome c-reporter fusion protein", "modified cytochrome c protein or functional analogue thereof derived from wild type cytochrome c", "targets mitochondria", and "reduced ability to induce apoptosis" are vague and unclear making the subject-matter of claim 1 and numerous dependent claims unclear (Article 6 PCT).

Firstly, it is not defined what is considered to by "wild type cytochrome c". Without the reference to a specific sequence, the definition of the mutations according to claims 5-10 does not seem to be clear. Furthermore, not all wild type cytochromes have the required function of initiating apoptosis. For example, yeast cytochrome c does not initiate apoptosis, whereas the highly homologous horse cytochrome c has said activity (see e.g D2, page 13034, left-hand column, last paragraph). Hence, claim 1 should clearly define the "wild type sequences".

Secondly, the form of the modification is not clear in claim 1. The modification is intended to be defined by the desired outcome, namely the modified cytochrome c "targets mitochondria" and "has a reduced ability to induce apoptosis in living cells". Said "definition" is not suitable, since it is only based on functional features, wherein said functional features are even not clear (see "targets" and "reduced").

Thirdly, the second part of the fusion protein, namely the marker, is only indirectly mentioned, namely in the form of "cytochrome c-reporter fusion protein". This is not sufficient.

Finally, the term "functional analogue" (see e.g. claim 1 and 11) is unclear, since it is not clear, which function is meant and to which extent said function must be preserved.